The Enzymic Hydrolysis of Steroid Conjugates

1. SULPHATASE AND β-GLUCURONIDASE ACTIVITY OF MOLLUSCAN EXTRACTS

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The application of sulphatase preparations to the hydrolysis of steroid sulphates has been mainly confined to substrates in which the sulphate radical is linked to the steroid through a phenolic hydroxyl group.

In a review of the literature concerning sulphatases, Fromageot (1938, 1950) classified these enzymes into four groups according to the nature of the substrate decomposed. Evidence was presented for a high degree of specificity of the enzyme towards the substrate. The systems most widely studied have been those active in hydrolysing phenolic (aryl) sulphates, but incapable of splitting those linked through the alcoholic (alkyl) hydroxyl groups such as occur in the neutral 17-oxosteroids (Lieberman & Dobriner, 1951). Thus Butenandt & Hoffstetter (1939) used Taka diastase to hydrolyse oestrone sulphate, and Cohen & Bates (1949) used myalase P to split the conjugated oestrogens of pregnant-mare urine. It was not found possible to effect the hydrolysis of the neutral 17-oxosteroid sulphates with sulphatase preparations of microbiological origin (Beuhler, Katzman & Doisy, 1950) or with Taka diastase of fungal origin (Stitch & Halkerston, 1953*a*). The ability of certain sulphatase preparations of molluscan origin to hydrolyse some neutral steroid sulphates has, however, been described recently (Henry, Thevenet & Jarrige, 1952; Henry & Thevenet, 1952; Stitch & Halkerston, 1953*a*, *b*). These workers also confirmed the findings of Dodgson & Spencer (1953*b*) that the extracts are a very rich source of β-glucuronidase. Further studies of the molluscan sulphatase by Roy (1954, 1956) and by Savard, Bagnoli & Dorfman (1954) have shown that it is not a general alkylsulphatase, but is specific for 3β-sulphates of 5α- and Δ^5-steroids.

This paper describes some enzymic properties of molluscan extracts, with special reference to the hydrolysis of steroid conjugates.

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EXPERIMENTAL

Reagents

Ether. A.R. grade ether was redistilled from ferrous sulphate.

Ethanol. This was purified for the Zimmermann reaction according to the method described by Cook, Dell & Wareham (1955).

Carbon tetrachloride. A.R. grade CCl_4 was fractionated through a six-pear column; the middle fraction was collected for use.

Substrates

Sodium androst-5-en-3β-ol-17-one sulphate (dehydro*epi*androsterone sulphate). This was prepared from androst-5-en-3β-ol-17-one (dehydro*epi*androsterone) (m.p. 148° uncorrected) by treatment with pyridine–sulphur trioxide (Talbot, Ryan & Wolfe, 1943). The m.p. was 191° uncorrected.

Sodium androsterone sulphate. The sample used was made available through the generosity of Dr R. Dorfman, of the Worcester Foundation, U.S.A. The m.p. was 165–180° uncorrected.

Sodium oestrone sulphate. An impure sample only was available.

Sodium pregnanediol glucuronide. Two preparations were used; one (m.p. 270–271°, not free from ketonic material) was supplied from the M.R.C. steroid reference collection. The second preparation was extracted from pregnancy urine by the method of Venning (1937), and was recrystallized from 90% ethanol (m.p. 269–270° uncorrected, not free from ketonic material).

Borneol glucuronide. The preparation used was supplied through the generosity of Professor J. K. N. Jones (Queen's University, Kingston, Ontario).

Sodium phenolphthalein β-glucuronide. This substrate was prepared according to the method described by Fishman, Springer & Brunetti (1948). The m.p. was 210°.

Preparation of the enzyme powder

Material. The limpets (*Patella vulgata*) were collected in batches of 10–20 kg. (3000–6000 limpets) from the Bristol Channel coast in the neighbourhood of Clevedon, Somerset. The majority of these molluscs were between 18 and 29 mm. in diameter, with a mean weight of 2·5 g. The common periwinkles (*Littorina littorea*) were obtained from the Marine Biological Station, Plymouth.

The limpets were processed in 2 kg. batches. They were ground in a mincer with a small amount of solid CO_2 powder and the mince was extracted at once with 2 l. of water in a ball mill for 30 min. After milling, the material was allowed to stand for 5–10 min. and the suspended protein and silt was decanted from the heavier shell fragments. The decanted suspension was centrifuged at 2000 rev./min. (M.S.E. major centrifuge) for 15 min. and the turbid supernatant stored at 5°. The residue and shell fragments were again extracted in the ball mill with 2 l. of distilled water for a further 15 min. The separation of shell fragments was repeated and the supernatant added to the first extract. For maximum yields the extraction may be repeated a third time with 1 l. of water. The combined supernatants were then brought to 65% (v/v) acetone concentration. Acetic acid was added dropwise during vigorous stirring until flocculation occurred (pH 6·0–6·5), and the precipitate was allowed to settle overnight at 5°. The supernatant acetone was removed and the powder was washed repeatedly with acetone at the centrifuge until desiccation was complete. The last traces of the solvent were removed *in vacuo*.

The powder (200 g.) was stirred with 10 l. of water for 1 hr. The insoluble material was removed by centrifuging at 1500 rev./min. for 30 min., and the slightly turbid supernatant stored at 5°. The insoluble residue was re-extracted with 7 l. of water by stirring for 15 min. After centrifuging, the supernatant was added to the first extract, and the residue discarded. The combined extracts were brought to pH 4·5 (glass electrode) by the addition of acetic acid and the precipitate was separated by centrifuging at 1500 rev./min. for 10–20 min. and discarded. Acetone was added to the supernatant to give a final concentration of 65% (v/v) and the mixture vigorously stirred. If flocculation did not occur, the pH was adjusted to approximately 6·5 with N-NaOH during stirring. The precipitate was allowed to settle overnight at 5° and the acetone removed. The precipitate was suspended in water (500–1000 ml.) and dialysed in Visking tubing against tap water for 18–24 hr., and finally against distilled water at 5° for 24 hr. Insoluble material removed by centrifuging was discarded and the supernatant was freeze-dried. This material is referred to throughout this paper as 'the enzyme'. The periwinkles were treated similarly.

Enzymic hydrolysis of substrates

The steroid substrates were incubated with the enzyme in 5 ml. of acetate buffer solution at 37° ± 0·1° in glass centrifuge tubes (15 ml.), and the liberated steroids extracted with ether. In the case of sodium pregnanediol glucuronide the incubation medium was made alkaline by addition of 1 ml. of 10% Na_2HPO_4 before extraction, since the glucuronide is itself soluble in ether at low pH values. Three extractions with 5 ml. of ether were carried out, and the combined extracts washed once with water. The ether was dried over anhydrous Na_2SO_4 for 3–4 hr., and the ether decanted and the Na_2SO_4 washed twice with 3 ml. of ether. The combined ether extracts and washings were then evaporated to dryness and the last traces of solvent removed *in vacuo*.

The free 17-oxosteroids liberated from dehydro*epi*androsterone sulphate and from androsterone sulphate were estimated colorimetrically by means of the Zimmermann reaction as modified by Callow, Callow & Emmens (1938).

Free pregnanediol was estimated by incubating the dried ether extract with 2 ml. of conc. H_2SO_4 at 25° for 20 min., the yellow colour being read after a further 45 min. in a Spekker absorptiometer with 0·5 ml. cells of 1 cm. light-path and Ilford violet filters.

Estimation of sulphatase activity

The sulphatase activity of the enzyme powders was determined by estimating the rate of release of dehydro*epi*androsterone from the sulphate under the optimum conditions of pH and substrate concentration (pH 4·8; substrate concentration approximately 0·21 mM; see below). One unit of activity is defined as that which releases 1 μg. of dehydro*epi*androsterone from the sulphate under these conditions.

One kilogram of limpets (approx. 300) yields 2–10 g. of powder assaying 800–2800 units/g. On purification the yield per kg. of limpets is more constant, typical values lying between 1 and 2·5 g. with a potency of 8000–12 000 units/g. The overall yield was 25 000–30 000 units/kg. of whole limpets.

The extracts prepared from *L. littorea* gave similar values for potency with a rather higher yield per kg. of molluscs than from *P. vulgata*. This would be expected owing to the absence of the large muscular foot.

Estimation of the β-glucuronidase activity of the enzyme powders

During the early stages of the work, the ability of the enzyme to liberate glucuronic acid from borneol glucuronide was utilized to demonstrate β-glucuronidase activity. The hydrolysis of borneol glucuronide was demonstrated by measuring the rate of release of glucuronic acid from the substrate in terms of D-glucuronolactone (Folin & Wu, 1919).

Phenolphthalein β-glucuronide, when available, was used as substrate for the assay of β-glucuronidase, the assay being simpler and more specific than that with borneol glucuronide. The method adopted has been described by Jarrige & Henry (1952), and the units of β-glucuronidase activity quoted in this paper were assessed under the conditions described by these authors.

The crude acetone-dried powders of *P. vulgata* assayed between $0·2 \times 10^6$ and $0·6 \times 10^6$ units/g. with phenolphthalein β-glucuronide as substrate, and the purified preparations varied between $1·0 \times 10^6$ and $2·0 \times 10^6$ units/g. These values are some twenty times the potency of the commonly available mammalian β-glucuronidase preparations. The yield per kg. of limpets was approximately $5·0 \times 10^6$ units.

RESULTS

According to Talbot *et al.* (1943) a spontaneous hydrolysis of dehydroandrosterone sulphate occurs in aqueous solution. To test this, a solution (1 mg./ml.) was maintained at room temperature for 10 days. It was then thoroughly shaken to distribute insoluble free steroid, and 2 ml. portions were taken for estimation of the free 17-oxosteroid. Some decomposition (approx. 3%) of the steroid conjugate occurred under these conditions. For this reason, only small quantities of the substrate solution were prepared as required and stored below 0°.

Table 1. *Hydrolysis of some steroid sulphates by the enzyme*

Steroid sulphate (400 μg.) was incubated with the enzyme (approximately 100 units of sulphatase in 10 mg. of powder), in 5 ml. of 0·2 M acetate buffer, pH 4·8; temp. 37° ± 0·1°.

Substrate	Enzyme (mg.)	Incubation time (hr.)	Steroid released (μg.)
Androsterone	—	1	7
Androsterone	—	5	5
—	10	1	4*
—	10	5	8*
Androsterone	10	1	6
Androsterone	10	5	5
Dehydro*epi*androsterone sulphate	—	1	4
Dehydro*epi*androsterone sulphate	—	5	5
Dehydro*epi*androsterone sulphate	10	1	100
Dehydro*epi*androsterone sulphate	10	5	194
Oestrone sulphate	—	1	8
—	10	1	4*
Oestrone sulphate	10	11	47

* Apparent steroid released.

Sulphatase activity of enzyme powders

A pH of 4·8 and a substrate concentration of about 0·21 mM appear to be optimum, giving a linear time–hydrolysis relationship up to 2 hr. and a linear enzyme concentration–hydrolysis rate up to an enzyme concentration of 2 mg. of freeze-dried material/ml. of incubation medium (Fig. 1).

The results, with various steroid sulphates as substrate, shown in Table 1, indicate that after 1 hr. 25 % of dehydro*epi*androsterone sulphate had been hydrolysed, and after 5 hr., 48·5 %. In 1 hr., 12 % of oestrone sulphate was hydrolysed. When androsterone sulphate was used as substrate no release of 17-oxosteroid could be demonstrated even after 5 hr. Other conditions of pH and substrate concentration were not investigated in relation to the hydrolysis of androsterone sulphate and oestrone sulphate.

Effect of some ions and of acetate buffer. Table 2 shows that both fluoride and phosphate ions (in a concentration of 0·01 M) inhibit the reaction strongly, whereas sulphate at a concentration of 0·02 M caused a 50 % inhibition of the sulphatase. Chloride and barium ions appeared to be without marked effect. Roy (1956), however, reported that 0·05 M chloride caused a 25 % inhibition of his sulphatase preparation.

Incubation of the substrate and enzyme with varying concentrations of acetate buffer at pH 4·8 showed that increase in the acetate concentration increased the rate of hydrolysis, with a maximum in the region of a final acetate incubation molarity of

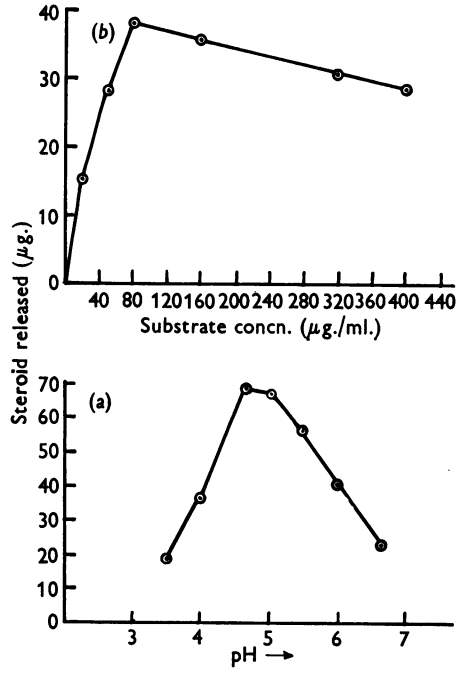

Fig. 1. Sulphatase activity of enzyme powder. Dehydro-*epi*androsterone sulphate was incubated at 37 ± 0·1°: (*a*) substrate concentration 400 μg./ml., for 2 hr. with 10 mg. of the enzyme in acetate buffer of required pH (final concentration 0·02 M); (*b*) substrate (20–400 μg./ml.) with 10 mg. of the enzyme for 1 hr. in acetate buffer (0·02 M final concentration).

0·2 M. There was no measurable hydrolysis when the enzyme was omitted at any of the acetate concentrations employed (Table 3).

β-Glucuronidase activity of enzyme powders

A pH of 3·5–4·0 appeared to be optimum; there was a linear relationship for at least 2 hr. between the rate of hydrolysis and substrate concentration up to 48×10^{-5} M.

Effect of barium on the hydrolysis of phenolphthalein β-glucuronide. An enzyme powder assayed $1{\cdot}0 \times 10^6$ units of β-glucuronidase/g. by the standard method. In a parallel incubation in 0·1 M barium chloride, the same powder assayed $1{\cdot}23 \times 10^6$ units/g. Barium was therefore shown not to be an inhibitor of the enzyme with respect to this substrate.

Enzymic hydrolysis of pregnanediol glucuronide. The hydrolysis of a steroid glucuronide was demonstrated with pregnanediol glucuronide. The optimum conditions for this enzymic reaction in 0·1 M acetate buffer were found to be approximately pH 3·5 and substrate concentration of 0·8 mg./ml.

Effect of dialysis on the sulphatase and β-glucuronidase activities of the molluscan extracts

Soda & Egami (1941) described the removal of glucosulphatase inhibitors from crude extracts of tropical marine molluscs (*Charonia lampas*). The crude extracts (i.e. of the acetone-dried powders) used here have shown a regular three- to four-fold increase in sulphatase activity on dialysis against distilled water (Table 4). This indicated the presence of a dialysable inhibitor in the crude extracts.

Dialysis appeared to have no effect on the β-glucuronidase activity under these conditions.

Table 2. *Effect of some common ions on the rate of hydrolysis of dehydroepiandrosterone sulphate by the enzyme*

The sulphate (0·21 mM) was incubated for 1 hr. at 37°±0·1° with the enzyme (approximately 130 units of sulphatase in 10 mg. of powder), in 5 ml. of 0·2 M acetate buffer at pH 4·8. Inorganic compounds were added.

Substance	Concn. (M)	17-Oxosteroid liberated (μg.)	Hydrolysis (% value without salt)
None	—	130	100
Na_2SO_4	0·002	123	94
	0·01	77	59
	0·02	57	44
NaCl	0·01	136	105
	0·02	120	92
	0·20	122	94
NaF	0·01	22	17
	0·02	2	1·5
	0·20	2	1·5
NaH_2PO_4	0·002	20	15
	0·01	0	0
	0·02	0	0
$BaCl_2$	0·01	134	103
	0·10	137	105

Table 3. *Effect of acetate buffer concentration on the hydrolysis of dehydroepiandrosterone sulphate by the enzyme*

The sulphate (0·21 mM) was incubated for 1 hr. at 37°±0·1° with the enzyme (approximately 130 units of sulphatase in 10 mg. of powder), in 5 ml. of acetate buffer, pH 4·8, of various concentrations.

Acetate buffer (final molarity)	pH of solution before incubation	Steroid released (μg.)
0·02	4·81	75
0·04	4·76	82
0·10	4·76	128
0·30	4·76	147
0·40	4·76	140
0·60	4·77	142

Table 4. *Effect of dialysis on the sulphatase (dehydroepiandrosterone sulphate) and β-glucuronidase (phenolphthalein β-glucuronide) activities of enzyme powders from* Patella vulgata *and* Littorina littorea

Crude enzyme powder (1 g.) was suspended in 50 ml. of water overnight at 5°. Water (50 ml.) was added, centrifuged, and portions of the supernatant were assayed by the standard methods described in the text. Crude powder (1 g.) was also suspended in 50 ml. of water in 20 mm. of Visking tubing and dialysed against 2½ l. of water overnight at 5°. The solution was centrifuged and portions of the supernatant were assayed by the standard methods.

Extract	Treatment	Sulphatase (units/g.)	β-Glucuronidase (10^6 units/g.)
Powders prepared from *P. vulgata*			
Sample 1	Non-dialysed	790	—
	Dialysed	1533	—
Sample 2	Non-dialysed	1500	0·4
	Dialysed	5000	0·39
Sample 3	Non-dialysed	1070	0·20
	Dialysed	2100	0·20
Powder prepared from *L. littorea*			
Sample 1	Non-dialysed	1800	—
	Dialysed	4500	—

Effect of pH *change during preparation of the enzyme extracts*

Dodgson & Spencer (1953*a*) have shown that β-glucuronidase from *P. vulgata* is irreversibly inactivated at pH 2·25 without appreciable loss in phenol-sulphatase activity (with potassium *p*-acetylphenyl sulphate as substrate).

The stability of the sulphatase and β-glucuronidase in the pH range 1·0–12·0 was studied. The results shown in Fig. 2 indicate that, although the sulphatase is inactivated in the alkaline range (over pH 9·0), it is much less affected by pH values between 2·0 and 3·0 than the β-glucuronidase, which is completely inactivated at pH 2·0.

DISCUSSION

Roy (1954) has achieved by paper electrophoresis a separation of the phenolsulphatase activity of extracts of *P. vulgata* from the steroid sulphatase with dehydro*epi*androsterone sulphate as substrate but was unable to separate glucosulphatase and steroid sulphatase activities. Extracts made from *L. littorea*, shown to be richer in glucosulphatase than those from *P. vulgata* (Dodgson & Spencer, 1953*b*), have, in our experience, no greater activity in hydrolysing dehydro*epi*androsterone sulphate than those of *P. vulgata*; this does not support the view that the glucosulphatase and steroid sulphatase are identical. Roy (1954) has found discrepancies in the quantitative effect of inhibitors on the steroid sulphatase of *P. vulgata* when compared with their effect on the glucosulphatase of *C. lampas* (Soda, 1936).

The marked specificity of the enzyme (Savard *et al.* 1954; Roy, 1956) limits the application for the hydrolysis of urinary steroid conjugates. It is, however, likely that the enzymic hydrolysis of the important urinary steroid metabolite dehydro*epi*androsterone sulphate may be of some value, as transformation of this compound readily occurs during hot acid hydrolysis.

The extracts made from *P. vulgata* have a β-glucuronidase activity about twenty times the potency of most preparations made from mammalian sources. This allows the use of very high concentrations of enzyme to overcome urinary inhibitors without the disadvantage of excessive protein. Cook *et al.* (1955) were able to add 200 000 units of β-glucuronidase to 25 ml. of urine for the hydrolysis of urinary corticosteroids with only 100 mg. of dried-enzyme preparation.

The significance of these findings with regard to (*a*) the yields of urinary steroids obtained after enzymic hydrolysis of urinary steroid conjugates, and (*b*) the utility of molluscan extracts for the enzymic hydrolysis of certain steroid conjugates for clinical purposes, is at present under investigation.

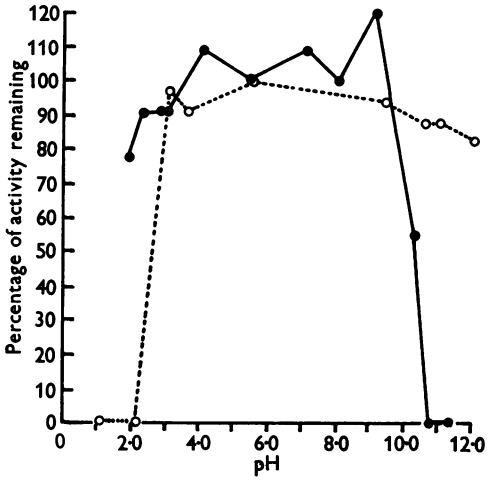

Fig. 2. Effect of pH on the stability of the sulphatase and β-glucuronidase prepared from *P. vulgata*. Purified enzyme powder (50 mg.) was shaken with 0·1 N-KCl (10 ml.), and adjusted to the required pH value with N-KOH or N-HCl. After 1 hr. the sulphatase and β-glucuronidase activities of the solutions were estimated by means of the standard methods described in the text. —, Sulphatase activity with dehydro*epi*androsterone sulphate; - - - -, β-glucuronidase activity with phenolphthalein β-glucuronide.

SUMMARY

1. Methods have been described for the preparation and purification from molluscs of a sulphatase capable of hydrolysing the sodium salt of dehydro*epi*androsterone sulphate, and a β-glucuronidase enzyme system.

2. Some properties of the sulphatase have been studied and a method of assay is described.

3. The sulphatase had no action on androsterone sulphate.

4. Some properties of the β-glucuronidase have been described with respect to its action on borneol glucuronide and pregnanediol glucuronide.

5. Evidence for the existence of a dialysable sulphatase inhibitor in the extracts has been presented. An irreversible inactivation of the sulphatase at pH values over 9·0 and of the β-glucuronidase at pH values below 3·0 has been demonstrated.

6. The specificity of the sulphatase is a limiting factor in the application to the hydrolysis of urinary steroid conjugates.

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2. HYDROLYSIS OF STEROID CONJUGATES IN URINE

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That urinary androgenic substances might be excreted as conjugates was first indicated by Funk, Harrow & Lewja (1929). Since that date, only two types of steroid conjugate have been isolated from urine—the glucuronides and the sulphates.

It has long been recognized that the drastic conditions of acid hydrolysis necessary to release steroids from these water-soluble derivatives in urine may result in losses or chemical transformations. Mild forms of acid hydrolysis have also been tried, but those steroids linked with glucuronic acid require for hydrolysis the stronger acid conditions which are responsible for the production of artifacts. These problems have been reviewed by Pincus (1954), and by Birke & Plantin (1954).

The successful use of bacterial glucuronidase for the hydrolysis of steroid glucuronides was reported by Buehler, Katzman, Doisy & Doisy (1949). Several authors (e.g. Cohen, 1951; Corcoran, Page & Duston, 1950; Cox & Marrian, 1951; Kinsella, Doisy & Glick, 1950) have since demonstrated that hydrolysis of urinary steroid conjugates with preparations of β-glucuronidase liberated considerably more formaldehyde-producing material from urine than hydrolysis and extraction at pH 1. More recently, Katzman, Straw, Buehler & Doisy (1954) have described the action of bacterial β-glucuronidase preparations on urinary oestrogen conjugates. A study of the hydrolysis of steroid conjugates by calf-spleen β-glucuronidase was made by Cohen (1951). Bitman & Cohen (1951) compared the hydrolysis of conjugated 17-oxosteroids by the acetate-buffer technique of Talbot, Ryan & Wolfe (1943), by calf-spleen β-glucuronidase and by hydrochloric acid at pH 1.

This paper describes the application of sulphatase and β-glucuronidase of molluscan origin to the hydrolysis of some urinary neutral 17-oxosteroid sulphates and glucuronides.

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